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Larvicidal activity of piperovatine and dichloromethane extract from *Piper corcovadensis* roots against mosquitoes *Aedes aegypti* L.

[Actividad larvicida de piperovatina y extracto de diclorometano de las raíces de *Piper corcovadensis* contra mosquitos vectores del dengue *Aedes aegypti* L.]

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Abstract: The research of new substances capable of controlling the *Aedes aegypti* mosquito is urgent due to the increase in the transmission of the diseases such as dengue, chikungunya and Zika virus by the vector. Thus, the aim of this study was to evaluate the larvicidal activity of crude extract of *Piper corcovadensis* roots, a native plant from Brazil, and of the isolated compound piperovatine against larvae of *A. aegypti* by the larval immersion test. The lethal concentration that killed 50% (LC_{50}) and 99% (LC_{99}) of larvae was determined by Probit analysis. The results indicated high larvicidal activity on A. aegypti larvae for crude extract of *Piper corcovadensis* roots with LC_{50} of 4.86 µg/mL and LC_{99} of 15.50 µg/mL and piperovatine with LC_{50} of 17.78 µg/mL and LC_{99} of 48.55 µg/mL. This work opens new perspectives to the development of future products with crude extract of *Piper corcovadensis* roots and piperovatine that can be applied to mosquito control.

Keywords: *Piper corcovadensis*; Piperovatine; *Aedes aegypti*; Larvicidal; Mosquito

Resumen: La investigación de nuevas sustancias capaces de controlar el mosquito *Aedes aegypti* es urgente debido al aumento en la transmisión de enfermedades como el dengue, el chikungunya y el virus Zika por el vector. Por lo tanto, el objetivo de este estudio fue evaluar la actividad larvicida del extracto crudo de las raíces de *Piper corcovadensis*, una planta nativa de Brasil, y del compuesto aislado piperovatine contra larvas de *A. aegypti* mediante la prueba de inmersión larvaria. La concentración letal que mató al 50% (LC_{50}) y al 99% (LC_{99}) de larvas se determinó mediante análisis Probit. Los resultados indicaron una alta actividad larvicida en larvas de *A. aegypti* para extracto crudo de las raíces de *Piper corcovadensis* con LC_{50} de 4.86 µg/mL y LC_{99} de 15.50 µg/mL y piperovatine con LC_{50} de 17.78 µg/mL y LC_{99} de 48.55 µg/mL. Este trabajo abre nuevas perspectivas para el desarrollo de futuros productos con extracto crudo de las raíces de *Piper corcovadensis* y piperovatine que pueden aplicarse al control de mosquitos.

Palabras clave: *Piper corcovadensis*; Piperovatine; *Aedes aegypti*; Larvicida; Mosquito

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INTRODUCTION

Aedes aegypti (Diptera; Culicidae) mosquito is a global public health problem mainly in tropical and subtropical countries, by the fact that it is the main vector of transmission of viruses that cause dengue, chikungunya and Zika virus. These diseases affect the population of several age groups causing mortality and morbidity with serious complications, with enormous impacts economic and on the quality of life of the individual (Pan American Health Organization 2017).

The main way to avoid the transmission of the diseases quoted is the control of the transmitting mosquito, which is done with organophosphates. However, there are limitations such as the development of chemical resistance by the mosquito and environmental pollution (Braga & Valle, 2007; Nunes *et al*., 2018).

Therefore, the development of new, more efficient, eco-friendly and safe inseticides, that also do not produce any adverse effect to non-target organisms, has been being stimulated (Pinto *et al*., 2016; Hari & Mathew, 2018). In this way, the larvicidal activity of plants is being investigated, since they are important natural sources of chemical compounds with countless biological activities, including the insecticide activity (Mendes *et al*., 2017; Mitić *et al*., 2018).

Piper genus is the largest in the family Piperaceae with approximately 1000 species with uses in traditional and medicinal in various parts of the world (Scott *et al*., 2008). Species of the *Piper* genus present several groups of secondary metabolites with important medicinal properties and the uses in pest control and as insecticides (Mgbeahuruike *et al*., 2017). These plants present piperamides with great insecticidal activity. These compounds are known to act as neurotoxins in the insect (Scott *et al*., 2008).

Piper corcovadensis (Miq.) C. DC. (Piperaceae), popularly known as "João brandinho" or "Falso-jaborandi", is a native plant from Brazil (Facundo *et al*., 2004). In folk medicine it is used for the treatment of rheumatism, influenza and cough (Parmar *et al*., 1997). The essential oil extracted from *P. corcovadensis* leaves and aqueous extract obtained from the residual plant material after hydrodistillation showed larvicidal activity against *A. aegypti* with LC_{50}=30.52 µg/mL and LC_{50}=2.93 µg/mL, respectively (Silva *et al*., 2016). Some compounds were identified in the *P. corcovadensis* extracts as the amides: piperovatine, piperlonguminine, corcovadine, isopiperlonguminine, isocorcovadine and chingchengamide; flavonoids: 3′,4′,5,5′,7-penta methoxyflavone, 3′,4′,5,7- tetra methoxyflavone, 5-dihydroxy-3′,4′,5′,7-tetra methoxyflavone and caffeic acid (Costa & Mors, 1981; Facundo *et al*., 2004). Piperovatine presents several reported biological activities such as acaricidal on *Rhipicephalus microplus* (Fernandez *et al*., 2018), leishmanicidal against *Leishmania amazonensis* (Rodrigues-Silva *et al*., 2009), tripanossomicidal (Veiga-Santos *et al*., 2013), antimicrobial (Silva *et al*., 2009), antifungal (Marques *et al*., 2007), antimycobacterial (Cunico *et al*., 2015) and piscicidal (McFerren & Rodriguez, 1998). Thus, the objective of the present study was to evaluate the larvicidal activity of the crude extract of *P. corcovadensis* roots and the isolated compound piperovatine on *A. aegypti* by the larval immersion test.

MATERIALS AND METHODS

Plant material, preparation of extract and isolation

Roots of *Piper corcovadensis* (Piperaceae) were collected in Ecological station of Caiuá (52º49' to 52º53' W and 22º34' to 22º37' S), Diamante do Norte, Paraná, Brazil. The voucher specimen was deposited in Herbarium of Nupélia (HNUP) under the number 16706. The preparation of crude extract of *P. corcovadensis* roots (CEPC) and the isolation of piperovatine was previously published by Fernandez *et al*. (2018). The CEPC was extracted in a Soxhlet apparatus using dichloromethane. For isolation of piperovatine, CEPC was submitted to column chromatography on silica gel 60 (0.063-0.2 mm, Macherey-Nagel) and eluted with chloroform, chloroform: ethyl acetate, ethyl acetate and methanol. The chloroform: ethyl acetate 80:20 fraction was submitted to column chromatography on Sephadex LH-20 (25-100 µ, Sigma-Aldrich®) and eluted with methanol and ethyl acetate.

Larvicidal activity on Aedes aegypti

Larval immersion test

The eggs of *A. aegypti* were provided by the *Laboratório de Transmissores de Hematozoários* of *Fundação Oswaldo Cruz*, Rio de Janeiro City, Rio de Janeiro, Brazil. The eggs were placed into standing water for hatching. The larvae hatched after 24 h and were fed with fish feed for growth until the third

stage. Third stage larvae of *A. aegypti* were then used for bio-tests.

The extracts of *P. corcovadensis* roots and piperovatine were diluted in aqueous solution with 2% ethanol at concentrations from 1.00 to 40.00 µg/mL. The positive control was prepared with organophosphate-based Temephós® at 400 mg/mL and the two negative controls which consisted of distilled water and an aqueous solution of 2% ethanol. Ten third stage larvae of *A. aegypti* were taken with Pasteur pipette and placed into 250 mL flasks with 10 mL solution at different sample concentrations (Costa *et al*., 2005; Bonato *et al*., 2010). The number of dead larvae was obtained after exposition to different concentrations for 24 h. Larvae that did not respond to stimuli were considered dead (Bonato *et al*., 2010). All tests were performed in triplicate and the larvae mortality was determined as following: LM = [(dead larvae x 100) / (total larvae)].

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and differences among averages, determined by Tukey's test at 5% significance level. Lethal concentrations (LC) to kill 50% (LC_{50}) and 99% (LC_{99}) of larvae and their respective 95% confidence intervals (CI) were calculated by Probit Analysis using the software Statistica version 7.0 Trial (StatSoft. Tulsa. OK. EUA).

Results and discussion

Piperovatine (Figure No 1) was isolated from CEPC by column chromatography and identified according to data previously published in Fernandez *et al*. (2018). Costa & Mors (1981) also identified piperovatine as a compound of *P. corcovadensis* root extract, as well as Facundo *et al*. (2004).

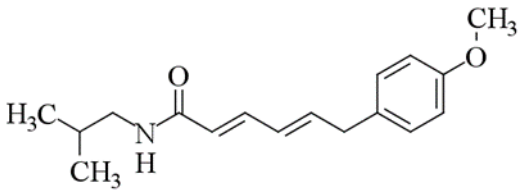

Figure Nº 1
Structure of piperovatine

The larvicidal activity of the CEPC and piperovatine against *A. aegypti* was investigated and the results are presented in Table No 1. The CEPC showed 100% larval mortality with concentrations from up to 10 µg/mL, while piperovatine presented 100% larval mortality at 40 µg/mL. In the CEPC concentration range from 7.50 µg/mL to 2.50 µg/mL, the mortality varied from 66.82% to 14.09%, whereas for piperovatine (30.00 µg/mL to 7.50 µg/mL), the mortality varied from 89.44% to 9.55%. As can be observed in the concentration-mortality curve for the larvae of crude extract of *P. corcovadensis* roots and piperovatine (Figure No. 2). The negative controls did not show larval mortality and the positive control presented 100% mortality of mosquito larvae at the tested concentrations.

LC_{50} and LC_{99} are presented in Table No. 2. The CEPC was more active with LC_{50} 4.86 µg/mL and LC_{99} 15.50 µg/mL, while piperovatine showed LC_{50} 17.78 µg/mL and LC_{99} 48.55 µg/mL. The probit model was adjusted to the data ($p \leq 0.05$) (Table No. 2). According to the classification of Cheng *et al*. (2003), compounds with LC_{50}<50 µg/mL are highly active against *A. aegypti* larvae. Thereby, CEPC and piperovatine have high activity on the larvae of the dengue mosquito.

In addition, the CEPC was more active than piperovatine, probably because plant extracts contain a number of chemical constituents, which, when tested, may have synergistic effects among the active principles, due to the different classes or structures contributing to the same activity (Maciel *et al*., 2002).

The present study is the first report about larvicidal activity of crude extract of *P. corcovadensis* roots and piperovatine on *A. aegypti*

larvae. However, Silva *et al*. (2016) evaluated the larvicidal activity against *A. aegypti* of the essential oil extracted from *P. corcovadensis* leaves and aqueous extract obtained from the residual plant material after hydrodistillation. The essential oil presented LC_{50} of 30.52 µg/mL and aqueous extract LC_{50} of 2.93 µg/mL. The essential oil showed strong ovoposition deterrent effect in 5 µg/mL with 38.1% laid eggs, inhibited egg laying of pregnant females and was able to interfere with the activity of proteases from L4 trypsin-like gut enzymes.

Table No. 1
Mean mortality of *Aedes aegypti* larvae treated with different concentrations of crude extract of *Piper corcovadensis* roots and piperovatine

Concentration µg/mL	% Average mortality	
	CEPC	Piperovatine
40.00	100.00^{a} ± 0.00	100.00^{a} ± 0.00
30.00	100.00^{a} ± 0.00	89.44^{b} ± 0.79
25.00	100.00^{a} ± 0.00	70.00^{c} ± 0.00
20.00	100.00^{a} ± 0.00	61.25^{d} ± 1.77
15.00	100.00^{a} ± 0.00	52.78^{e} ± 3.93
10.00	100.00^{a} ± 0.00	20.00^{f} ± 0.00
7.50	66.82^{b} ± 4.50	9.55^{g} ± 0.64
5.00	45.00^{c} ± 7.07	0.00^{h} ± 0.00
2.50	14.09^{d} ± 5.79	0.00^{h} ± 0.00
1.00	0.00^{e} ± 0.00	0.00^{h} ± 0.00
Positive control	100.00 ± 0.00	
Negative Control	0.00 ± 0.00	

CEPC: crude extract of *Piper corcovadensis* roots; Mean ± standard error; Averages followed by the same lower letter in the column, do not differ among themselves by Tukey's test ($p \leq 0.05$)

Several species of the genus *Piper* have been being investigated regarding their insecticidal activity, including the larvicidal activity against *A. aegypti*. Kanis *et al*. (2013) investigated the extract of *P. ovatum* roots on *A. aegypti* larvae, which exhibited LC_{50} 2.9 µg/mL and LC_{99} 6.1 µg/mL, whereas the standardized extract with piperlonguminine showed LC_{50} 2.1 and LC_{99} 4.1 µg/mL. Marques *et al*. (2017) also evaluated the larvicidal activity of *Ottonia anisum* against *A. aegypti*. The crude hexane extract presented 100% larval mortality at a concentration of 200 µg/mL. The methanolic extract of leaves of *P. nigrum* showed larvicidal activity against dengue vector of LC_{50} 34.97 µg/mL and significantly reduced the activities of α- and β- carboxylesterases and superdioxide (Lija-Escaline *et al*., 2015).

The results of this study are in line with others that also investigated the potential of amides against *A. aegpyti*. Greger (1984) reports that studies by SAR indicates that the N-isobutylamine moiety might play a crucial role in the larvicidal activity of isobutylamides. Other studies related that piperamides act as neurotoxins in the insect, and lipid amides can modified axonal excitability by an effect upon sodium currents, described as ''pyrethroid-like'' (Scott *et al*., 2008).

Table Nº 2
LC_{50} and LC_{99} of the crude extract of *Piper corcovadensis* roots and piperovatine against *Aedes aegypti* larvae

	Inclination ± SE	LC_{50} ± SE (µg/mL)	95% CI (LCI-UCI)	LC_{99} ± SE (µg/mL)	95% CI (LCI-UCI)	$\chi 2^{(df=30)}$	p-value
CEPC	4.62 ± 0.72	4.86^{b} ± 0.42	4.01-5.70	15.50^{b} ± 0.92	11.78-25.06	5.5	1.000
Piperovatine	5.32 ± 0.65	17.78^{a} ± 0.57	15.71-19.88	48.55^{a} ± 3.82	39.50-67.02	5.6	1.000

CEPC: crude extract of *Piper corcovadensis* roots; SE: Standard error; LC_{50}: lethal concentration that kills 50% of the exposed larvae; LC_{99}: lethal concentration that kills 99% of the exposed larvae; LCL: lower confidence limit; UCL: upper confidence limit; $\chi 2$: Chi-square; df: degree of freedom; p: probability; Negative control: aqueous solution of 2% ethanol; Positive control: Temephós (400 mg/mL). Averages followed by the same lower letter in the column, do not differ among themselves by Tukey's test ($p \leq 0.05$)

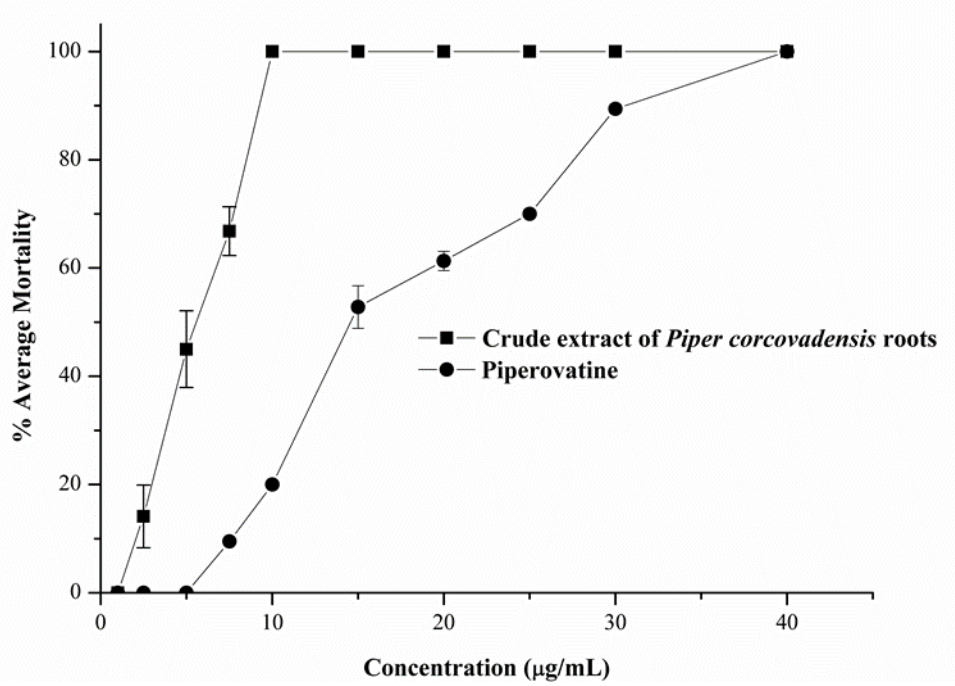

Figure Nº 2
Concentration-mortality curve for the larvae of the *Aedes aegypti* of crude extract of *Piper corcovadensis* roots and piperovatine

According to Maleck *et al*. (2014), the piperlonguminine isolated of *P. tuberculatum* showed LC_{50}=12 µg/mL in L3 (third instar) and in L4 (fourth instar) larvae of *A. aegypti* caused changes to the digestive tube cells. In other hand, the piplartine (piperamide), also isolated from *P. tuberculatum*, showed action with LC_{50}=155.5 µg/mL, and in L4 larvae delayed the dengue mosquito development. Park *et al*. (2002) evaluated the larvicidal action of isobutylamides isolated from *P. nigrum* fruit on L3 larvae of *A. aegypti*. After 48h after of treatment, the effect was more pronounced in retrofractamide A (LC_{50}=0.039 µg/mL) than in pipercide (LC_{50}=0.1 µg/mL), guineensine (LC_{50}=0.89 µg/mL), and pellitorine (LC_{50}=0.92 µg/mL). Piperine (LC_{50}=5.1 µg/mL) was relatively ineffective.

Therefore, the roots extract of *P. corcovadensis* and piperovatine showed promising results that can be used in the control of the mosquito *A. aegypti*.

CONCLUSIONS

The crude extract of *P. corcovadensis* roots and piperovatine showed a high larvicidal potential against *A. aegypti*. Thus, this study opens new perspectives to the development of future products employed on the control of dengue mosquito. However, further studies are needed in different stages of development from the mosquito, as well as studies about the effect on non-target organisms and the residual effect on the environment.

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